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Phenotypic correlates of *Clock* gene variation in a wild blue tit population: evidence for a role in seasonal timing of reproduction

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Abstract

The timing of reproduction in birds varies considerably within populations and is often under strong natural selection. Individual timing within years is dependent on a range of environmental factors in addition to having an additive genetic basis. In vertebrates, an increasing amount is known about the molecular basis for variation in biological timing. The *Clock* gene includes a variable poly-glutamine (poly-Q) repeat influencing behaviour and physiology. Recent work in birds, fish and insects has demonstrated associations between *Clock* genotype and latitude across populations, which match latitudinal variation in breeding time. In this study, we investigated the phenotypic correlates of variation in *Clock* genotype within a single blue tit *Cyanistes caeruleus* population over two successive breeding seasons. In females, but not in males, we observed a general trend for birds with fewer poly-Q repeats to breed earlier in the season. Incubation duration was shorter in both females and males with fewer repeats at the polymorphic *Clock* locus. Poly-Q *Clock* allele-frequency was homogenously distributed within the study population and did not exhibit any consistent environment-related variation. We further tested for effects of *Clock* genotype on reproductive success and survival, and found that females with fewer poly-Q repeats produced a higher number of fledged offspring. Our results therefore suggest that (i) selection in females, but not in males, for fewer poly-Q repeats may be operating, (ii) the across–population associations in timing of breeding involving this locus could be linked to variation within populations, and (iii) the *Clock* gene might be involved in local adaptation to seasonal environments.

Keywords: breeding, circadian clock, natural selection, poly-glutamine, polymorphism

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Introduction

Most organisms inhabit environments that vary in time and in space. Particularly at temperate latitudes, these environments show marked seasonal variation, with changes over many orders of magnitude in productivity. Consequently, organisms have evolved mechanisms that enable them to track the variation in their environment, in order to exploit critical resources most effectively (Scheiner 1993; Merilä *et al*. 2001a, b; Schultz & Kay 2003). A well-established ecological model for studying the consequences of decisions about seasonal timing is the date at which a female bird begins to lay eggs (Perrins 1970). In small passerines, where females lay large clutches of eggs that will hatch into rapidly growing offspring, it is critical that they time reproduction appropriately to coincide with the peak in food availability for their offspring (Grieco *et al*. 2002; Visser *et al*. 2005). Long-term studies of wild populations have shown that timing of reproduction is often under strong selection within populations: those females that time their reproduction to coincide with the peak in food availability produce a greater share of the next generation (e.g. Sheldon *et al*. 2003; Charmantier *et al*. 2008). In recent years, quantitative genetic analyses of these populations have shown that part of the variation in timing of breeding has an additive genetic basis (e.g. van der Jeugd & McCleery 2002; Sheldon *et al*. 2003; McCleery *et al*.

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2004; Nussey *et al*. 2005). Despite some degree of heritability, variation in timing of breeding within populations can be considerable, both between years, and within years, with differences between individuals within years being dependent on a range of intrinsic and extrinsic spatial and environmental factors, such as individual age, the timing of local phenology of tree species, altitude, and population density (e.g. Wilkin *et al*. 2006).

A wealth of research into the molecular genetic basis of rhythms in animals has led to the characterization of genes that control important aspects of the daily and annual cycle. One well-understood system is the molecular genetic basis for the circadian clock, for which conserved genes that regulate biochemical oscillations with a period of 24 h have been described for a range of vertebrates and invertebrates. In vertebrates, the *Clock* gene (positive circadian regulator) includes a variable polyglutamine (poly-Q) repeat. The poly-Q region of the *Clock* gene serves as a transcriptional trans-activation domain (Gekakis *et al*. 1998) that influences behaviour and physiology in experimental studies of both mice and *Drosophila*. Recent work on birds (Fidler & Gwinner 2003; Johnsen *et al*. 2007), *Drosophila* (Tauber & Kyriacou 2005) and fish (O'Malley & Banks 2008) suggests the possibility that large-scale environmental variation in timing (e.g. timing of reproduction, timing of dispersal/migration) is underpinned by genetic systems. For example, repeat length variation in *period*, a *Drosophila* circadian clock gene, is maintained by balancing selection for adaptation of the fly circadian clock to differing ranges of ambient temperature at different latitudes (Tauber & Kyriacou 2005). Our study is mainly motivated by the results of a recent avian cross-population comparison (Johnsen *et al*. 2007), which suggests an association between variation in the length of the poly-Q repeat in blue tits *Cyanistes caeruleus* with latitude: a higher mean repeat number was observed at higher latitudes. A similar latitudinal cline in average allele length at this variable locus of the *Clock* gene was recently demonstrated in Chinook salmon *Oncorhynchus tshawytscha* (O'Malley & Banks 2008). Birds such as blue tits show considerable variation in timing of breeding associated with latitudinal variation, and given the findings of Tauber & Kyriacou (2005) and Johnsen *et al*. (2007), it is possible that variation at the *Clock* gene might contribute to this variation.

The *Clock* protein poly-Q repeat motive and its flanking sequences is conserved within Aves and this conservation seems to limit poly-Q repeat length variation (Fidler & Gwinner 2003; Johnsen *et al*. 2007). This poly-Q length variation is maintained in several bird species but with different frequencies (Fidler & Gwinner 2003; Johnsen *et al*. 2007), which might be indicative that the length variation at this particular region of the *Clock* gene itself is selectively advantageous (Wren *et al*. 2000; Johnsen *et al*. 2007). Studies in *Drosophila* have shown that length variation in the poly-Q region can affect binding affinity of the *Clock* transcription factor and thus alter the circadian phenotype (Darlington *et al*. 1998).

While cross-population analyses show that allele frequency and latitude (and hence, presumably, environmental features) covary, to our knowledge, no study has investigated within-population *Clock* gene variation in relation to the timing of seasonal events. Furthermore, there has been no attempt, so far, to quantify the fitness consequences of carrying particular *Clock* genotypes in relation to reproductive success or survival within populations. In this study, we test a series of hypotheses derived from Johnsen *et al*.'s (2007) finding of a latitudinal gradient in mean allele length in blue tits. First, we hypothesize an association between *Clock* allele length and seasonal timing within populations. Second, we tested whether, within populations, longer mean allele length at the *Clock* locus was associated with breeding in habitats, or locations, which were on average phenologically later with respect to seasonal timing. Third, we asked whether any effect of *Clock* poly-Q genotypes on timing of reproduction depends on the sex of the bird; as seasonal timing is thought generally to be a female-driven process in small passerine birds, any association should be more pronounced in females (Ball & Ketterson 2008; Caro *et al*. 2009). Our study allows us to test genotypic frequencies with respect to realized fitness within a replicate wild population. Hence, if the latitudinal gradient in allele length is currently maintained by selection, then we predicted that, within this population, mean *Clock* allele length should be subject to stabilizing selection.

Our research focused on a population of blue tits in Wytham, Oxfordshire (all birds from the 2006 and 2007 breeding populations were analysed), where detailed life-history data has been collected since 2001. This blue tit population was also sampled in the study of Johnsen *et al*. (2007), which revealed five alleles at the *Clock* poly-Q locus present within the population subset (in a sample of 96 individuals). Our study objectives were thus: (i) to further characterize variation at the *Clock* gene in the Wytham blue tit population; (ii) to determine how this variation relates to phenotypic variation in seasonal timing; (iii) to ask whether there is any association between environmental variation and an individual's genotype; and, finally (iv) to determine whether any selective benefit is conferred by the possession of a particular genotype.

Materials and methods

Study population, sampling and fitness measures

Approximately 1200 nest-boxes with 250–450 breeding pairs of blue tits per year are monitored as part of an ongoing study on the breeding ecology of tits in Wytham

Woods, Oxfordshire, UK (51°47′N, 1°20′W). Each year, all reproductive attempts are monitored from nest-building until fledging of young, parents are caught and identified, and offspring marked with metal rings. Individual marking permits estimation of individual fitness measures such as the number of offspring fledged and number of offspring recruited to the breeding population in the following year, and annual survival of adults. Blood samples were collected, under licence, by brachial venepuncture, and stored in SET buffer (0.15 M NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0). In this study, we analysed blood samples from 979 blue tit male and female breeders collected in 2006 and 2007, all of which were captured between day 6 and day 14 of the nestling phase (hatch day = day 0, where hatch date is defined as the date of first hatching in cases of asynchronous hatching), and thus sampled while breeding. While there is no evidence that males influence the timing of reproductive decisions (i.e. egg-laying) of the pair, any understanding of genetic variation within the population requires that we consider the frequency and behaviour of allelic variants in the sex where the phenotype is not expressed, and thus, we complemented the number of samples from female breeders with analysis of male partners in both years. Further, analysis of associations between timing and male genotype ought to provide a null model comparison with females.

Genomic DNA was extracted using a standard ammonium acetate protocol and samples stored at –80 °C. A total of 950 successfully genotyped samples over two consecutive years were included in the analysis of the associations between *Clock* genotype, timing of breeding and environmental parameters (out of the 979 samples analysed in total, 21 samples from unsuccessful breeding attempts plus 8 samples from which we could not amplify the *Clock* gene were excluded; see Results section for more details). These comprised 256 females and 203 males in 2006, and 283 females and 208 males in 2007; there were 481 individual females and 370 individual males in the analysis (hence, 57 females and 42 males were sampled in both years of the study; the analysis of phenotypic variation corrects for these repeated measures; see below).

Analysis of Clock *poly-Q alleles*

Genomic DNA samples were analysed for length polymorphism in the poly-Q repeat of the *Clock* gene. To screen for putative additional variation with respect to the previously published blue tit sequences at the *Clock* locus in focus (Johnsen *et al*. 2007), we determined the *Clock* genotype for blue tit females breeding in 2006 by polymerase chain reaction (PCR) amplification of the variable poly-Q repeat in the blue tit (corresponding to human *Clock* gene exon 20; Steves *et al*. 1999). We used a 10-μL reaction volume using the primer set previously described for sequencing of the *Clock* poly-Q alleles (Johnsen *et al*. 2007), while PCR protocol and temperature profiles were adapted according to Johnsen *et al*. (2007). Amplified products were prepared for sequencing using QIAGEN MinElute 96 UF PCR Purification Kits and QIAVac Multiwell vacuum manifolds. Nucleotide sequences of purified PCR fragments were determined with the BigDye Terminator ready reaction mix, version 3.1 (Applied Biosystems) under standard sequencing conditions according to the manufacturer's protocol. Unincorporated dye terminators were removed by isopropanol precipitation. The reaction products were detected on an ABI PRISM genetic analyser 3100 (Applied Biosystems), and edited using Sequencher 4.8 (GeneCodes). To screen genomic DNA samples of male breeders in 2006 and both male and females breeders in 2007, we adopted the 6-FAM labelled primer combination design and amplification protocol previously published (Johnsen *et al*. 2007). Amplification products (2 μL) were resolved using an ABI PRISM genetic analyser 3100 (Applied Biosystems) and a molecular size standard (GeneScan-500 LIZ, Applied Biosystems). To calculate amplification product sizes, we used GeneMapper 3.7 (Applied Biosystems) alongside with three positive control samples of known *Clock* genotype (number of poly-Q repeats was determined by sequencing reaction). 99.2% (971/979) of all blue tit samples were successfully genotyped. We used the repeated samples (57 females and 42 males) to assess the repeatability of the genotyping process, which was done blindly in each year with respect to the results of the previous year's analysis. In 98/99 cases, the genotype was identical for the repeated samples; for one female, one allele was scored as differing by one repeat across the two samples. Hence, the error rate was low.

Ecological genetic analysis

Calculation of allelic frequency data and tests for deviation from Hardy–Weinberg equilibrium (HWE) were performed using GenePop version 4.0 (Raymond & Rousset 1995) with the following Markov chain parameters: dememorization 10 000; batches 10 000; iterations per batch 10 000.

Test for allelic dominance. We used one-way ANOVA (analysis of variance) to test for allelic dominance for the most common *Clock* alleles. Timing parameters [lay date (LD), observed hatch (OH)] were standardized for each year (e.g. standardized LD (LD_{stand}) was calculated as follows: $LD_{stand} = (LD\text{-}LD_{(average\ per\ year)})/SD$ (with SD as the standard deviation of LD per year); incubation duration (ID) was defined as OH–(LD + CS) (with CS as clutch size). We compared standardized timing parameters to detect the type of gene effect model (additivity, dominance) for birds that were heterozygous or homozygous for one of the two most common *Clock* alleles (*Clk*polyQ$_{12}$ and *Clk*polyQ$_{13}$).

Due to power limitations, this approach can only be taken for the two most abundant alleles.

Clock *genotype.* To test for an association between *Clock* genotypes with varying length of poly-Q repeats and the timing of breeding, we define poly-Q *Clock* genotype as the sum of poly-Q repeats of both alleles (p + q); this analysis is equivalent to analysing the effect of mean allele length. This approach allows the combined effect of both alleles on the phenotype to be assessed. We also analysed the data with a full genotype model treating each genotype as different level (with genotype as random or fixed factor), providing the advantage to distinguish between individual *Clock* genotypes 13/13 and 12/14 (as number of poly-Q repeats). Further, to facilitate comparisons between studies, we analysed the data with blue tit genotypes classified in three categories of allele length, as defined in the study of Johnsen *et al*. (2007): (1) birds having two alleles *Clk*polyQ$_{12}$ or smaller (≤ Q12/≤ Q$_{12}$); (2) heterozygous birds with one allele *Clk*polyQ$_{12}$ or smaller and the other *Clk*polyQ$_{13}$ or larger (≤ Q12/≥ Q$_{13}$), and (3) birds with both alleles *Clk*polyQ$_{13}$ or larger (≥ Q13/≥ Q$_{13}$). 44.4% (378/851) of blue tits in this sample fall into category 1, 45.4% (386/851) into category 2, and 10.2% (87/851) birds into category 3. Results for all three genotype classification methods revealed similar results, and we opted for the use of a continuous variable representing *Clock* poly-Q genotypes and their effects on timing, unless indicated otherwise [in particular, the three categories as defined in Johnsen *et al*. (2007) were used for graphical representations of the effect of genotype on timing of reproduction].

Clock *genotype and timing of breeding.* We investigated the relationship between *Clock* genotypic variation and timing of breeding using general linear models (GLM) and general linear mixed models (GLMM) with normal error distribution with identity links, while correcting for appropriate fixed [age, sex, altitude, breeding density, year for models with incubation duration (relative measure not standardized by year) as explanatory variable] and random (ring number to account for repeated measures of individuals between years) effects based on the knowledge developed over the course of the population studies at Wytham. We tested for associations between *Clock* genotype and the following aspects of reproductive scheduling: (i) date of laying the first egg, or lay date (LD); (ii) observed hatch date (OH); (iii) incubation duration (ID). Together, these measures summarize important aspects of reproductive timing, all of which are under selection in this, or other, wild bird populations (e.g. Garant *et al*. 2007; Charmantier *et al*. 2008; B.C. Sheldon, unpublished data). In addition to the date of first egg-laying, we use two additional measures here. Incubation duration represents the interval between laying and hatching — this is often accelerated within populations when ambient temperatures are higher in order to maintain synchrony between birds and their food supply (e.g. Cresswell & McCleery 2003), and hence, we might expect that incubation duration could also be influenced by a latitudinal gradient. Lastly, while laying date has long been the focus of studies of timing of reproduction in birds, it could be argued that laying date is only important to the extent to which it leads to synchronization of peak food demand of nestlings with peak food supply (if one accepts that this is the key effect driving timing of breeding in these systems); hatching date is a better measure of this critical date. Timing parameters were standardized for each year as described earlier. Note that some statistical tests share the same data and are therefore not independent and the results should be interpreted with caution; the purpose of these analyses is to detect year-specific effects.

To investigate repeatability of timing traits in the population as a whole, we used mixed modelling to estimate the variance components of parental male and female contributions to the variability in timing of reproduction. To maximize power, we expanded our data set to the full set of life-history data of the focal blue tit population that have been collected since 2001. We analysed the relative impact of females and males in determining the pair's phenotype (i.e. life-history traits with respect to timing of reproduction) for breeding events where the identity of both parents was known, and where at least one parent bred successfully at least twice in its lifetime with a partner of known identity (n = 636 females, n = 586 males; number of breeding events: n = 974). A general linear mixed model was used for timing traits (standardized values) with no fixed effects, but with female and male ring number fitted as random effects in the model. P values were estimated by comparing the change in deviance between models with different assemblages of random effects; the obtained values were tested against the chi-square distribution, with one degree of freedom.

Clock *genotype and environmental variation.* To test for associations between environmental variables and genotype within the population, we employed two approaches. First, we modelled *Clock* genotypic variation as a function of several environmental variables known to be associated with variation in timing of breeding in tits, specifically: altitude, oak richness and breeding density (Wytham Woods is a heterogeneous woodland, divided into nine sectors showing different habitat characteristics (Minot & Perrins 1986), and breeding density was calculated per sector per year (Wilkin *et al*. 2006)). This approach allows us to test whether the distribution of specific alleles varies as a function of the environment, and whether any patterns detected are related to changes in the timing of breeding. Second, we asked whether there was any evidence for

spatial effects on allele-frequency distributions. This was done by calculating the spatial autocorrelation coefficient with respect to repeat-number at the *Clock* locus (e.g. van der Jeugd & McCleery 2002). The autocorrelation coefficient (r) ranges from –1 to 1. Significance of the spatial autocorrelation values was tested by constructing a two-tailed 95% confidence interval around the null hypothesis of no spatial genetic structure (i.e. $r = 0$). Spatial autocorrelation analysis was performed for 10 distance classes (99 random permutations) using GenAlEx (Peakall & Smouse 2006).

Clock *genotype and fitness measures.* We estimated selection on the alleles at the *Clock* locus directly, by regressing (i) fecundity-related fitness measures (specifically: number of fledglings and number of recruits in the following year), and (ii) survival to breeding on genotypic values of individuals. Because in both years a part of the breeding population was involved in a cross-fostering experiment including brood size manipulation (S.C.L. Knowles, unpublished data), we restricted our analyses of fitness to unmanipulated broods ($n = 521$) and control nests where no brood size enlargement/reduction was made ($n = 105$). Control nests within this experiment did not differ from unmanipulated nests with respect to an effect of *Clock* poly-Q genotype on fitness measures ($F_{1,625} = 0.38$, $P = 0.542$, $n = 626$; regression coefficient (SE): 0.0219 (0.0353)), and were therefore included in our analyses; this resulted in a total of 626 individuals included in our fitness analyses ($n = 351$ females, $n = 275$ males). Standardized selection differentials measuring the effect of genotype on fitness were obtained by regressing fitness (relative number of fledged individuals, calculated as number of individuals fledged for a breeding event divided by the mean fitness of the population) on the standardized value of *Clock* genotype [calculated as (individual value-mean)/SD, i.e. the standardized normal deviate].

We further tested for an effect of *Clock* poly-Q repeat numbers on adult survival (life-history data from 2006 to 2008 only justify estimating local survival, defined as the probability of recapture as a breeding bird in the study are in the following year) using GLM with binomial error distribution with logit-link function, where an interaction of genotype*sex, sex, age and year of the breeding event were fitted as additional explanatory variables. As binomial distributions often display higher or lower variability than predicted by a null model, we estimated the dispersion parameter φ to control for over- ($\varphi > 1$) or under-dispersion ($\varphi < 1$) of the data. We used the estimated dispersion parameter whenever $\varphi > 1$ to adjust for the residual degrees of freedom (d.f.) to the value of the residual variance; whenever under-dispersion occurred, φ was set at 1. In order to describe the pattern of selection at a population level, we performed fitness and survival analyses for both males and females together and both sexes separately. In analyses for fitness effects and survival to the next breeding season, we used standardized values of genotype (mean = 0, SD = 1), we also fitted quadratic genotype values to the model to test for stabilizing selection. In general, the expectation from observing latitudinal gradients is that, if these are currently maintained by selection, we should observe stabilizing selection at the population level. Unless stated otherwise, analyses were carried out using GenStat 11 (VSN International Ltd).

Results

Within-population variation in the Clock *gene in the blue tit*

The total sample size for our study was $n = 979$ blue tit breeders in 2006 and 2007. We successfully amplified and sequenced the *Clock* poly-Q variable length region of all female blue tits breeding in 2006 ($n = 261$). Genomic DNA of male breeders in 2006 ($n = 204$) and both female ($n = 294$) and male ($n = 212$) breeders in 2007 was successfully genotyped for variation at the poly-Q locus of the *Clock* gene. For unknown reasons, we could not obtain a genotype for eight blue tit samples (genomic DNA extraction and PCR amplification, both by means of sequencing and genotyping were independently repeated twice). Unsuccessful breeding attempts (2006 $n = 5$, 2007 $n = 15$) were excluded from analyses with respect to timing of reproduction, but considered as part of the breeding population, and thus, included for genotype frequency analyses (breeding population: $n = 971$). We controlled for repeated observations in cases where an individual bird successfully bred in both years ($n = 99$ individuals) by including a random effect for individual identity. Thus, the final data set including all successful breeders in 2006 and 2007 was $n = 950$ (i.e. $n = 851$ individual birds, 99 of which successfully bred in both years). When years were analysed separately, we avoided pseudoreplication of double breeders by randomly choosing one sample and designating it as breeding in one of the two years; therefore, each individual appears only once in year-specific analyses (all successful breeders in 2006 and 2007 only included once: $n = 851$).

Amplified products were identical except with respect to the number of poly-Q repeats and correspond to the previously published blue tit *Clock* sequences (GenBank Accession nos AY338423–338428, AY376853 and DQ026514–15). We identified six length variants of *Clock* poly-Q alleles in the Wytham blue tit population (*Clk*polyQ$_{10,11,12,13,14,16}$; subscript indicates the number of poly-Q repeats), with alleles of a length of 11–14 poly-Q repeats found at medium to high abundance. The two most common alleles (*Clk*polyQ$_{12,13}$) accounted for 88% of allelic diversity (Table 1). The frequency distribution of alleles is consistent

Table 1 *Clock* poly-Q allele frequencies and observed heterozygosity (*H*) for the blue tit population used in this study (double breeders only included once). Allele frequencies of the complete breeding population of 2006 and 2007 in Wytham Woods are compared with the subset from the Wytham population included in the cross-population comparative study of Johnsen *et al*. (2007)

Population	*n*	Q_{10}	Q_{11}	Q_{12}	Q_{13}	Q_{14}	Q_{15}	Q_{16}	*H*
2006 + 2007 (both sexes)	851	0.005	0.064	0.600	0.279	0.042	0	0.009	0.565
Females (2006 + 2007)	482	0.006	0.062	0.593	0.290	0.041	0	0.008	0.562
Males (2006 + 2007)	369	0.004	0.070	0.617	0.262	0.047	0	0.011	0.568
Johnsen *et al*. (2007) (Wytham Woods)	100	0.010	0.057	0.589	0.307	0.036	0	0	0.521

Table 2 General linear mixed models testing the effect of *Clock* poly-Q genotype on timing of reproduction: females and males from both years are analysed separately (double breeders are controlled for by including bird ring number as random effect). The table shows that all effects of *Clock* poly-Q genotype on timing of breeding are stronger in females, also compare with Fig. 1. Fixed effects included: *age, altitude, breeding density* (*sex* included for analyses on full data set; *year* is included for analyses on ID). For details of the full models including all the fixed effects see Table S2, Supporting information (LD_{stand} is standardized laying date, OH_{stand} is standardized observed hatch date, ID is incubation duration)

Data subset	Timing trait	*n*	*F*	d.f.	*P* value	Regression coefficient (SE)
2006 + 2007 (both sexes)	LD_{stand}	950	0.04	1	0.839	0.0058 (0.0284)
	OH_{stand}		0.71	1	0.399	0.0235 (0.0279)
	ID		7.54	1	0.006	0.1295 (0.0472)
Females (2006 + 2007)	LD_{stand}	539	1.56	1	0.212	0.0489 (0.0392)
	OH_{stand}		3.62	1	0.056	0.0726 (0.0382)
	ID		4.22	1	0.040	0.1352 (0.0658)
Males (2006 + 2007)	LD_{stand}	411	1.32	1	0.251	–0.0467 (0.0406)
	OH_{stand}		0.86	1	0.356	–0.0371 (0.0401)
	ID		2.86	1	0.092	0.1190 (0.0704)

Model: (p + q) + age + altitude + breeding density (+sex for full data set; +year for analyses on ID); ring number included as random effect.

with the distribution described for the subset of the Wytham population (in Johnsen *et al*. 2007: only *Clk*$polyQ_{16}$ was not scored in this subset), and did not differ between sexes (Table 1). *Clk*$polyQ_{9,15,17}$, three rare alleles (overall frequency below 0.001) recorded in the cross-population study by Johnsen *et al*. (2007), were not found in the Wytham blue tits sampled here.

Population allele frequencies were tested for deviation from Hardy–Weinberg equilibrium (HWE). Poly-Q allele frequency within the Wytham blue tit population did not deviate significantly from HWE, either overall (all blue tits genotyped with repeated breeders only included once; $P = 0.876$) or between years or sexes ($P > 0.331$). The observed overall heterozygosity is $H = 0.565$, and is similar in the two sexes ($H = 0.562$ females, $H = 0.568$ males). Blue tit breeding pairs ($n = 384$) mated randomly with respect to *Clock* genotype, as expected for a population in HWE ($r = 0.0013$, $P > 0.843$).

Test for allelic dominance. Tests for allelic dominance in two of the most common *Clock* alleles on a subset of birds either homozygous or heterozygous for any combination of these two alleles ($n = 742$, equivalent to 78% of the entire population) did not reveal any effects of dominance overall (Table S1, Supporting information), nor when the sexes were analysed separately ($P > 0.172$). Based on these results, we assume additivity of allelic effects in subsequent analyses.

Is there a connection between Clock *genotype and reproductive timing phenotype within the focal population?*

Clock *poly-Q genotypes.* *Clock* genotypes with varying length of poly-Q repeats were found to be significantly associated with timing parameters. When significant, fewer poly-Q repeats were always associated with either shorter incubation duration, earlier lay date or observed hatch date. Importantly, the results are year- and sex-specific, and are outlined in detail in Tables 2 and 3 (sexes analysed separately for each year).

We found that females, but not males, with fewer poly-Q repeats at the *Clock* locus [fewer poly-Q repeats at the variable *Clock* locus or genotype class 1 ($\leq Q12/\leq Q_{12}$)] have earlier hatch and lay dates (Table 3; Fig. 1A, B). This is a consistent pattern found both overall and for years

Table 3 A yearly level GLM analysis of the effect of *Clock* poly-Q genotype on timing of breeding (LD_{stand}, standardized laydate; OH_{stand}, standardized observed hatch; ID, incubation duration): years were analysed separately for both sexes, and double breeders randomly excluded. These yearly analyses indicate that the overall effect of *Clock* genotype on timing of reproduction among females (Table 2**)** is driven by stronger associations in 2007. Variables included are: *age, altitude, breeding density*. Significant values ($P < 0.05$) are presented in bold. For details of the full models including all the fixed effects, see Table S3, Supporting information

Data subset	Timing trait	*n*	*F*	d.f.	*P* value	Regression coefficient (SE)
2006 females	LD_{stand}	225	0.53	1	0.469	–0.0426 (0.0587)
	OH_{stand}		0.00	1	0.965	–0.0026 (0.0597)
	ID		3.01	1	0.084	0.1512 (0.0871)
2006 males	LD_{stand}	182	0.79	1	0.375	–0.0589 (0.0663)
	OH_{stand}		1.07	1	0.302	–0.0706 (0.0682)
	ID		0.57	1	0.450	0.0740 (0.0978)
2007 females	LD_{stand}	257	3.97	1	**0.047**	**0.1095 (0.0550)**
	OH_{stand}		4.58	1	**0.033**	**0.1085 (0.0507)**
	ID		0.85	1	0.357	0.0960 (0.1040)
2007 males	LD_{stand}	187	0.24	1	0.622	–0.0298 (0.0603)
	OH_{stand}		0.00	1	0.964	–0.0025 (0.0561)
	ID		2.54	1	0.113	0.1960 (0.1230)

Model: (p + q) + age + altitude + breeding density.

analysed separately (Fig. 1A, B), although the effect on LD is only statistically significant for 2007 (Table 3). All relationships between *Clock* poly-Q genotype and timing of breeding are stronger in females (Tables 2 and 3); we found a nonsignificant tendency for a sex*genotype interaction on LD ($F_{1,948} = 2.93$, $P = 0.087$, $n = 950$) and a significant interaction with respect to OH ($F_{1,948} = 3.94$, $P = 0.047$, $n = 950$), but not for incubation duration ($F_{1,948} = 0.03$, $P = 0.854$, $n = 950$). Analyses of both years separately revealed a stronger effect of *Clock* genotype on LD and OH in the year 2007 (Table 3), although no significant overall year*genotype interaction was found for any of the timing traits in focus (all results with $P > 0.213$).

We found a significant effect of *Clock* poly-Q genotype on ID, where shorter incubation times are significantly correlated with fewer poly-Q repeats (Table 2; Fig. 1C). This effect is largely driven by females, although a nonsignificant trend in the same direction is also present in males (Table 2; Fig. 1C). As incubation duration is a timing parameter closely linked with both LD and OH (see Methods for definition of ID), and it is often the case that birds breeding later have shorter incubation times (e.g. Both & Visser 2005), we controlled for that by adding lay date as a covariate to the model of *Clock* genotype on incubation duration. The significant effect of *Clock* poly-Q length on incubation duration is still present when standardized LD is included as additional covariate [*Clock genotype*: $F_{1,948} = 9.47$, $P = 0.002$, $n = 950$; regression coefficient (SE): 0.1472 (0.0478); *effect of lay date on ID*: $F_{1,948} = 12.70$, $P < 0.001$, $n = 950$; regression coefficient (SE): –0.1991 (0.0559); compare with results for ID of the complete data set in Table 2]. Hence, we view timing parameters (LD, OH) and incubation duration as two independent effects, specifically: an indication of a timing effect, and an effect linked to the duration of this part of the reproductive cycle. An alternative possibility is that the 'incubation duration' effect represents an effect of developmental time in the egg for the offspring (i.e. that it is an effect of the offspring's genotype rather than that of the two parents). We tested this by estimating development time [defined as observed hatch–(laying date + clutch size)] for each genotyped blue tit that had been ringed as a nestling. There was no relationship between this estimate of development time and genotype ($F_{1,253} = 0.86$, $P = 0.391$, $n = 254$; regression coefficient (SE): 0.068 (0.079). This result did not change when the sexes were analysed separately [*Females*: $F_{1,125} = 1.32$, $P = 0.191$, $n = 126$; regression coefficient (SE): 0.19 (0.14); *Males:* $F_{1,127} = 0.26$, $P = 0.793$, $n = 128$; regression coefficient (SE): –0.022 (0.085)].

Relative contribution of each sex to the pair's phenotype. Estimates of the relative importance of the female and male in determining breeding parameter traits of the pair clearly show that the timing of reproduction is largely determined by the female. The estimated percentage of variance explained by female and male of the breeding pair ($n = 974$) was 32% and 8% for standardized LD, respectively, 32% and 10% for standardized OH ($n = 971$), respectively, and 16% and 0% for ID ($n = 970$), respectively. To assess the statistical significance of these effects, we further contrasted models with either female or male random components with models where both male and female identities were included. There were no large differences between models when 'females only' were contrasted with models were

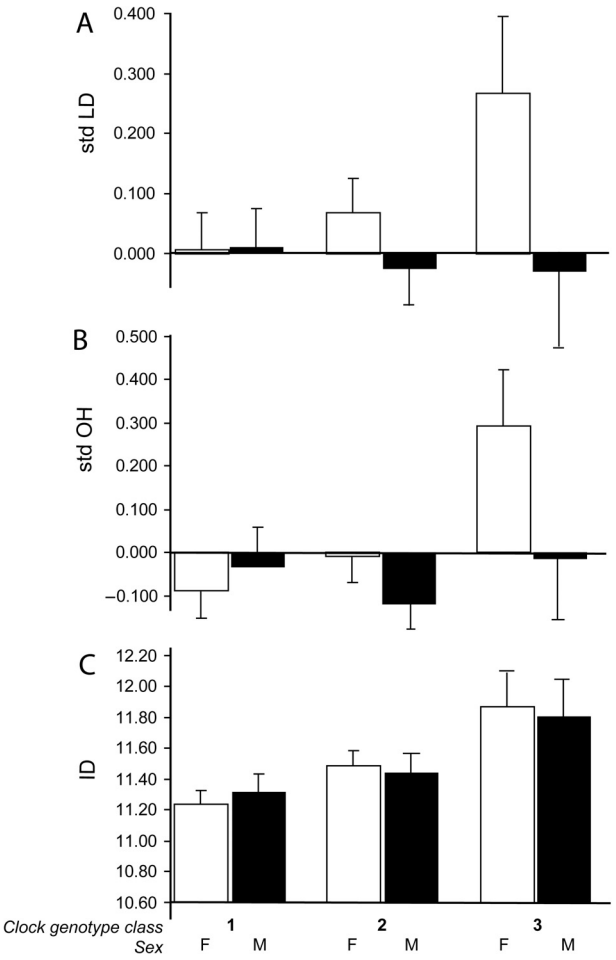

Fig. 1 Effect of *Clock* poly-Q genotype on timing of reproduction in female (white bars) and male blue tits (black bars) in 2006 and 2007. *Clock* genotype scored in classes following Johnsen *et al*. (2007): **1** ($\leq Q12/\leq Q_{12}$); **2** ($\leq Q12/\leq Q_{13}$); and **3** ($\geq Q13/\geq Q_{13}$). The average value of each timing trait is plotted for each *Clock* genotype class and sex separately. Life-history traits related to the timing of reproduction in focus here are: (A) date of the first egg, i.e. (standardized) lay date (LD); (B) observed hatch date (OH); (C) incubation duration (ID). (A) and (B) show that females, but not males, with a higher number of poly-Q repeats at the *Clock* locus have later hatch and lay dates. (C) indicates that longer incubation times are significantly correlated with higher numbers or poly-Q repeats, this trend is stronger in females but the pattern is also present in males. Timing traits are plotted as standardized values (zero mean, unit variance). Error bars represent standard errors.

both male and female identity were fitted as random effects ($\chi^2 = 2.63$, 3.91 and 0.35, $P = 0.100$, 0.048 and 0.440 for standardized LD, OH and incubation duration, respectively; but note the borderline significant effect for standardized OH). In contrast, significant differences were observed when models with male identity was fitted as random component and contrasted with a model with both male and female component fitted together ($\chi^2 = 37.42$, 35.68. 7.63, $P < 0.001$, 0.001 and 0.006 for standardized LD, OH and incubation duration, respectively). Hence, while females significantly contribute towards the variability of timing of reproduction, males have a much weaker effect on timing parameters.

Is there any association between environmental and genetic variation in the population?

Tests for spatial autocorrelation confirm that no significant spatial structure for the polymorphic *Clock* locus (i.e. no spatial autocorrelation) is present within the Wytham blue tit population. Correlation between matrices for genotype and geographical distance all fell within the confidence interval limits, both overall and for sexes analysed separately (*r* values did not differ from random for all 10 distance classes analysed; *r* ranged between –0.176 and 0.252 with a mean value of 0.018).

The study site, Wytham Woods, is heterogeneous in terms of altitude, oak tree abundance (within 50 m of the nest-box) and breeding density, and different areas are known to show some consistency in terms of timing of breeding. However, the spatial distribution of *Clock* genotypes across Wytham was found to be highly homogenous. It did not vary with respect to altitude, oak tree abundance or breeding density as a measure of environmental heterogeneity (*altitude*: $F_{1,950} = 0.20$, $P = 0.652$; regression coefficient (SE): 0.3578 (0.7932); *oak* tree abundance: $F_{1,950} = 0.41$, $P = 0.523$; regression coefficient (SE): –0.1364 (0.2075); *breeding density*: $F_{1,950} = 0.01$, $P = 0.909$; regression coefficient (SE): 0.0028 (0.0245)). Further, no genotype*environment interaction with respect to LD, OH and ID was found for altitude, habitat type, oak richness or breeding density as a measure of environmental heterogeneity (*clock genotype*altitude, LD*: $F_{1,950} = 0.58$, $P = 0.448$; *OH:* $F_{1,950} = 1.09$, $P = 0.296$; *ID:* $F_{1,950} = 0.72$, $P = 0.396$; *clock genotype*oak richness, LD*: $F_{1,950} = 1.30$, $P = 0.254$; *OH:* $F_{1,950} = 1.13$, $P = 0.288$; *ID:* $F_{1,950} = 1.23$, $P = 0.268$; *clock genotype*breeding density, LD*: $F_{1,950} = 2.41$, $P = 0.141$; *OH:* $F_{1,950} = 1.72$, $P = 0.190$; *ID:* $F_{1,950} = 0.01$, $P = 0.917$), neither overall nor for sexes analysed separately (data shown for overall analyses). In summary there is no suggestion of any small-scale environmental correlations with *Clock* genotype, compared to the large-scale patterns documented by Johnsen *et al*. (2007).

Clock *genotype and fitness measures — is there natural selection on allelic variants?*

Our data provide evidence for an effect of *Clock* poly-Q repeat number on the number of fledged offspring in females ($F_{1,349} = 6.64$, $P = 0.010$, $n = 351$) but not in males ($F_{1,273} = 0.30$, $P = 0.584$, $n = 275$): females with shorter *Clock* poly-Q alleles successfully raised larger numbers of

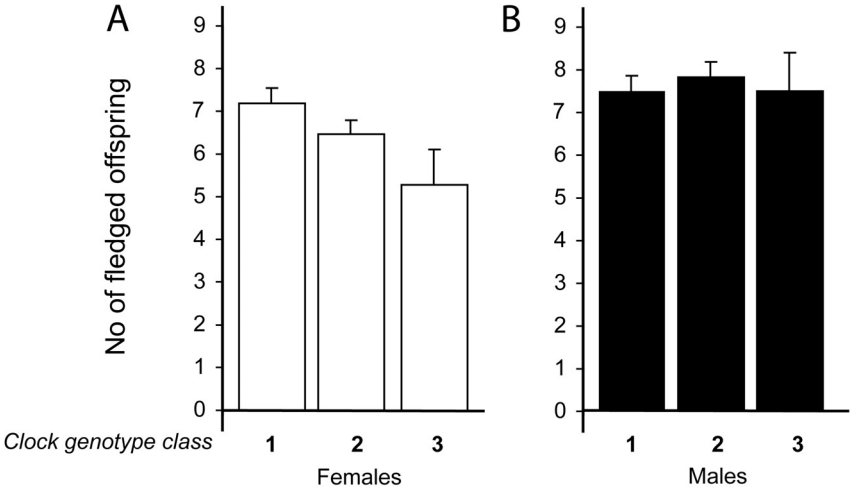

Fig. 2 Effect of *Clock* poly-Q genotype (genotype classes as in Fig. 1) on reproductive success in blue tits calculated as average number of fledged offspring for (A) females (white bars) and (B) males (black bars). Only unmanipulated nests and experimental controls of both years in focus are included ($n_F = 311$, $n_M = 249$). Our results indicate that females, but not males, with shorter *Clock* poly-Q alleles successfully raised higher numbers of offspring to the fledging stage. Error bars represent standard errors.

Table 4 GLMM testing for an effect of *Clock* poly-Q genotype (standardized values) on reproductive success (measured as number of fledged offspring; bird identity included as a random effect to control for repeated measures when years are pooled; only unmanipulated nests and experimental controls are included). We found that females, but not males, with fewer poly-Q repeats at the variable *Clock* locus successfully raised higher numbers of offspring to the fledging stage (also see Fig. 2). Fixed effects included are: *age, year of breeding*. *Ring number* included as random effect

	Females (2006 + 2007)					Males (2006 + 2007)				
Model	*n*	d.f.	*F*	*P* value	Regression coefficient (SE)	*n*	d.f.	*F*	*P*-value	Regression coefficient (SE)
$(p + q)_{stand}$	351	1	6.64	0.010	–0.7052 (0.2736)	275	1	0.30	0.584	–0.1492 (0.2720)
$[(p + q)_{stand}]^2$		1	1.80	0.180	0.2103 (0.1566)		1	1.17	0.193	0.1649 (0.1262)
Age		1	0.03	0.857			1	0.02	0.897	
Year		1	11.82	< 0.001			1	9.24	0.003	

Model: (p + q) + age + year of breeding; ring number included as random effect.

offspring to the fledging stage (Fig. 2, Table 4), largely due to differences in entire brood failure. The effect of *Clock* genotype on the number of fledged young remains significant when controlling for lay date ($F_{1,349} = 4.01$, $P = 0.046$, $n = 351$), suggesting a direct effect of *Clock* genotype on reproductive success. Taking the number of offspring recruited to the breeding population in the following year as a fitness measure, no significant effect of *Clock* poly-Q genotype was found, either overall or for sexes analysed separately ($P > 0.463$); however, dispersal is quite high in this species, and it is possible that testing the effects of *Clock* gene variation on more inclusive indicators of reproductive success (such as number of offspring recruited, or number of grand-offspring fledged) may require large sample sizes to provide definite answers. Our data thus suggest a fitness advantage for females with fewer poly-Q repeats at the variable *Clock* locus (i.e. lower p + q-value or genotype class 1) when fitness is evaluated at the offspring fledgling stage (Table 4). This result is consistent (i) with the effect of *Clock* genotype on timing of reproduction in the two sexes, with earlier hatch and lay dates being associated with fewer poly-Q repeats at the *Clock* locus in females but not in males, and (ii) with the fact that birds breeding earlier in the season are known to have higher fledgling success and recruitment rates (Verboven & Visser 1998). Selection differentials for the number of offspring fledged depending on parental *Clock* genotype are presented in Table 5. Additionally, we fitted quadratic terms for *Clock* genotype to these analyses, but found no effect on fledging success (Table 4). Hence, there is no evidence of stabilizing selection on the *Clock* genotype from these analyses.

Our data show no association between *Clock* poly-Q repeat numbers on adult survival to the following breeding season [for (p + q)*:* deviance ratio of genotype*sex interaction: 1.76, p = 0.185; deviance ratio for genotype (p + q) as explanatory variable: 1.64, $P = 0.200$; for $(p + q)^2$: deviance ratio of genotype*sex interaction: 0.42, $P = 0.517$; deviance

Table 5 Standardized selection differential of *Clock* poly-Q genotype on the number of fledged offspring. *Clock* genotype (calculated as p + q) has been standardized (mean = 0, SD = 1), in a data set where both sexes and both years are included (note that variance and mean of p + q for males and females are highly equivalent). Standardized selection differential (S) is calculated as a regression coefficient where: *Y* = number of fledged individuals (only unmanipulated nests and experimental controls are included; standardized by dividing the number of fledged young per nest by the average number of fledged in the data set); *X* = standardized *Clock* genotype [(value – mean)/SD)]. Significant values ($P < 0.05$) are presented in bold

Data subset	*n*	d.f.	Selection differential (*S*)	SE	*F*	*P* value
Females (2006 + 2007)	351	1	–0.0723	0.0343	4.46	**0.035**
Females (2006)	169	1	–0.0553	0.0453	1.49	0.224
Females 2007	182	1	–0.1006	0.0503	4.01	**0.047**
Males (2006 + 2007)	275	1	0.0025	0.0325	0.01	0.940
Males (2006)	137	1	0.0727	0.0437	2.77	0.098
Males 2007	138	1	–0.0682	0.0465	2.15	0.145

ratio for genotype $(p + q)^2$ as explanatory variable: 0.19, $P = 0.67$]. The GLMM treating ring number as a random effect did not converge; we thus used standard GLMs without controlling for the fact that each bird was represented twice in the analysis. Year- and sex-specific analyses (including both fitted single and quadratic standardized terms for *Clock* genotype) do not reveal an effect of *Clock* genotype on adult survival ($P > 0.206$).

Discussion

In this study, we investigated within-population *Clock* gene variation in a wild blue tit population with respect to the timing of breeding and quantified the fitness consequences of carrying particular *Clock* genotypes in relation to reproductive success. Previous work on this species demonstrated a latitudinal gradient with longer population mean allele length associated with higher breeding latitudes (Johnsen *et al*. 2007). Johnsen *et al*. (2007) suggest the latitudinal cline in poly-Q allele frequency they detected across blue tit populations, with shorter poly-Q alleles being more common at low latitudes, reflected local adaptation to latitudinal gradients in photoperiod parameters such as seasonal rate-of-change of photoperiod (Johnsen *et al*. 2007). Birds such as blue tits show considerable variation in timing of breeding associated with latitudinal and altitudinal variation (e.g. Visser *et al*. 2003), and given the recent findings (Johnsen *et al*. 2007) it is plausible that variation at the *Clock* gene underlies some of this variation. We found weak, but consistent evidence that *Clock* gene variation was associated with seasonal timing, in females only, and also evidence that this is under directional natural selection for some fitness components.

Our study is one of few studies that have explored behaviour-related genes in a free-living nonmodel species in the wild. Cross-population studies on blue tits (Johnsen *et al*. 2007) and Chinook salmon (O'Malley & Banks 2008) have reported a latitudinal cline in allele length and frequency of the polymorphic *Clock* gene with longer repeats found at higher latitudes. The latitudinal range in both studies is very large ($\Delta_{latitude} = 25.9°$ in Johnsen *et al*. 2007; $\Delta_{latitude} = 12.9°$ in O'Malley & Banks 2008). In blue tit populations investigated by Johnsen *et al*. (2007), the positive correlation between genotype and latitudinal gradient was only significant when one monomorphic population from the most southern latitude was included in the correlation, although the overall nonsignificant trend remained when that population was removed from the analysis; a cross-population comparison of bluethroats *Luscinia svecica* did not reveal a latitudinal cline with respect to the polymorphic *Clock* locus (Johnsen *et al*. 2007). In both studies on salmon and blue tits, the patterns of length variation between the different populations included in the cross-population comparison do not reflect those of neutral microsatellite markers, which suggest the possibility of balancing selection. The latitudinal cline in frequency of poly-Q repeat numbers suggests environmental selection based on functional effects of allele length. Both Johnsen *et al*. (2007) and O'Malley & Banks (2008) hypothesize that the observed clinal variation should reflect an adaptation to photoperiodic parameters correlated with latitude. Johnsen *et al*. (2007) further discuss the possibility that the fact that higher frequencies of longer *Clock* alleles are associated with higher latitudes could be related to the seasonal migratory polymorphism of blue tits at different latitudes with higher numbers of poly-Q repeats putatively associated with the propensity to migrate or disperse. However, for both blue tits and salmon, any mechanistic association between *Clock* poly-Q variation and circadian phenotypes remains to be identified. Establishing functionality of simple sequence repeats is very challenging, because those functional polymorphisms typically only operate on small, quantitative effects on phenotype (reviewed in Fondon *et al*. 2008).

We hypothesized that within populations, *Clock* gene variation should be maintained under stabilizing selection, influenced by specific features of the environment, such

that variation within populations is maintained by spatial and temporal heterogeneity. The distribution of *Clock* genotypes was found to be spatially homogenous (i.e. allelic homogeneity), and our data provide no indication for spatial effects on *Clock* allele frequency distributions (i.e. spatial autocorrelation) within the population. The fact that we do not observe any significant associations between environmental predictors and *Clock* poly-Q genotype suggests that a link between small-scale habitat choice and this variable locus is unlikely. Importantly however, we have found that the nonrandom distribution of birds with different *Clock* genotypes on a large geographical scale (Johnsen *et al*. 2007) can be translated into a framework of nonrandom timing of breeding associated with variation at a polymorphous locus of the *Clock* gene in females, at the scale of a single population.

Our study is the first attempt to analyse within-population patterns of *Clock* gene distribution with respect to timing of breeding. In parallel, we also investigated fitness consequences of particular *Clock* genotypes. In females, but not in males, we observed a general trend for birds with fewer *Clock* poly-Q repeat numbers to breed earlier in the season. The observed pattern is in agreement with a previous cross-population study on blue tits reporting a latitudinal cline with increasing frequencies of shorter *Clock* alleles with lower latitude, where breeding is earlier. Importantly, however, our study shows that the latitudinal cline in *Clock* genotypes described by Johnsen *et al*. (2007) can here be translated into an association between timing of breeding and *Clock* genotype in females, on a much smaller geographical scale (the size of the study area is just 388 ha). Finally, incubation duration in the focal population of our study was shorter in both females and males with fewer numbers of repeats at the polymorphic *Clock* locus. Hence, within a single population, variation at this locus seems to be related to several measures of reproductive timing.

The fact that our results reveal an effect of *Clock* gene variation on timing traits in females in a similar direction to a cross-population study (Johnsen *et al*. 2007) covering latitudinal distances of approximately 2800 km is striking if we consider that the scale of our focal population is so much smaller. Similarly, the variation in mean lay date observed over the entire latitudinal range of the species covers approximately 30 days (e.g. Visser *et al*. 2003), while the standard deviation of annual lay date within the Wytham population is approximately 6 days. Further, Johnsen *et al*. (2007) report nine different *Clock* alleles in 14 populations covered in their comparative study, six of which we identified in our study population. Our results thus highlight the relatively small scale at which effects of *Clock* locus be observed, and highlight the scope of within-population genetic variance at the variable *Clock* locus and the associated phenotypic consequences — not only as an effect on timing traits, but also reflected in reproductive success.

We further investigated the potential for sex differences in the relationship between *Clock* gene variation and timing of reproduction, and hypothesized that any link between timing of breeding and *Clock* genotype should be seen more strongly in females; our data confirm this. That this association is found only in females but not in males is consistent with analysis of the longer term data from this population which shows that females are the sex that most strongly determines the pair's phenotype with respect to timing of breeding. Our data further show a clear effect of *Clock* genotype on the speed of reproduction, where birds with fewer numbers of poly-Q repeats incubate eggs for shorter periods. Although stronger in females, this trend is also present in males. This association in males is hard to interpret and we can only speculate about putative explanations: one hypothetical explanation may be that the male behaviour towards the female during incubation could contribute to the speed of incubation time; this effect deserves further attention; note, however, that an effect of the male on this variable was not supported by our variance component estimates.

Analysis of fitness components (measured as the number of fledged offspring) in relation to genotype suggested that in females (but not in males), selection is currently targeting the *Clock* poly-Q locus with respect to timing of reproduction. Although our results suggest directional selection for shorter *Clock* poly-Q alleles, and the latitudinal cline is consistent with stabilizing selection, this is only true if the position of the cline is stable over time. Given recent changes in the timing of spring, and the general trend for earlier phenology in Western Europe (e.g. Parmesan & Yohe 2003), it is possible that the latitudinal cline might be undergoing a shift; analysis of historical specimens might be interesting in this regard. There is good evidence from a wide range of bird species, including multiple blue and great tit populations across Europe (Visser *et al*. 2003), that breeding time has undergone temporal shifts to earlier values in recent decades. Hence, it is possible that selection might be acting to shift the population mean genotype at this locus to smaller values, consistent with earlier average breeding time and faster reproductive cycles.

Given the observed general trend that females with shorter poly-Q *Clock* alleles breed earlier and fledge more offspring, we suggest that the polymorphic *Clock* locus is among a subset of genes in one regulatory cascade involved in shaping the phenotype of some life-history traits related to the timing of reproduction. Although not overall significant in our focal population, this pattern is consistent with the observation that populations at higher latitudes have a higher frequency of class 3 ($\geq Q13/\geq Q_{13}$) *Clock* genotypes (Johnsen *et al*. 2007). Behavioural traits are typically multigenic, heterogenous, weakly penetrant, and environmentally influenced, and thus, it is very difficult to

identify polymorphic loci influencing a particular phenotypic trait in wild populations. The power of identifying functional polymorphic alleles is limited by the magnitude of their effects. It is, therefore, worth noting that the sample size employed here is relatively large, and the effects seen were rather weak, equivalent to a difference of only *c*. 0.25 SD in timing measures across the range of commonly observed genotypes (e.g. Fig. 1); this is equivalent to of the order of 1.5 days difference in timing in this sample. While this difference is quite small, timing of breeding is frequently under quite strong natural selection; in the long-term study of the great tit at Wytham, the mean standardized selection differential on egg-laying date is –0.28 (Charmantier *et al*. 2008). A difference of the order of that due to the *Clock* genotype in the present study might be associated with a relative fitness difference of the order of 7–8% under such strong selection.

While the effect of *Clock* alleles on timing parameters such as egg-laying date and hatching date was statistically marginal in some cases (Fig. 1), we consider the observed pattern to be reliable for two reasons. First, the effect is seen in females and not in males (this would be expected for a phenotype in the context of timing of reproduction, and we have shown that the pair's phenotype is determined by the female in our study population). Second, the direction of the effect is as one would expect, considering the results of Johnsen *et al*. (2007): since birds breed earlier at lower latitudes (associated with fewer poly-Q repeat numbers), we would expect that shorter poly-Q alleles would be associated with earlier breeding times in the intrapopulation comparison reported here. Our results on environmental correlates with *Clock* poly-Q variation allow us to distinguish whether the association between *Clock* genotype and timing arises because of a direct effect of the genotype, or because birds with particular genotypes tend to settle in habitats that have characteristic timings. As we found no evidence for spatial or environment-linked variation in *Clock* genotype occurrence throughout the woodland based on the array of environmental variables investigated, our results suggest a direct association between *Clock* genotype and timing of reproduction.

While there is evidence for an additive genetic basis to timing of breeding in the related and ecologically similar species (great tit: see van der Jeugd & McCleery 2002 and McCleery *et al*. 2004; we are not aware of any studies in the blue tit), estimates of the heritability of timing of breeding are typically rather small (of the order of 0.10–0.20). Hence, while this is a character often under strong selection, and with a close link to the question of adaptation to variable environments, finding a molecular genetic signal in phenotypic variation can be expected to be challenging, and may require considerable statistical power, as is generally the case for fitness components in natural populations (Ellegren & Sheldon 2008).

In summary, we observed a general trend in female, but not in male blue tits for fewer poly-Q repeat numbers associated with earlier breeding times. Incubation duration was shorter in both females and males, with fewer numbers of repeats at the polymorphic *Clock* locus. On the level of reproductive success, we found that females with shorter poly-Q repeats produced a greater number of fledged offspring. Our data thus suggest that selection in females, but not in males, for a lower number of poly-Q repeats at the variable *Clock* locus may be operating. Although the overall pattern found in our study is consistent both in itself and in the context of previous cross-population results, the effect sizes are not very large. Thus, we advocate caution in terms of general inferences drawn from this study and encourage future studies to focus on the genetic structuring of *Clock* genes, as well as their phenotypic effects, at a local level in other species and populations.

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Author contributions

Study conception: M.L., B.C.S. Field work: M.L., S.C.L.K., M.J.W. Molecular analysis: M.L. Statistical analysis: M.L., M.S., B.C.S. Manuscript preparation: M.L., M.S., B.C.S.

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The authors have a broad interest in evolutionary biology, ecology, molecular and behavioural biology. In this study the authors focus on the evolutionary genetics of the timing of reproduction in a wild blue tit population using a candidate gene approach to link phenotypic traits to their underlying molecular genetic basis.

Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Test for allelic dominance on a subset of birds either homozygous or heterozygous for any combination of *Clk*polyQ$_{12}$ and *Clk*polyQ$_{13}$, the two most common alleles

Table S2 Details for all fixed effects included in the full general linear mixed models (presented in Table 2) testing the effect of *Clock* poly-Q genotype on timing of reproduction (density is breeding density)

Table S3 Details for all fixed effects included in the yearly-level general linear models (presented in Table 3), analysing the effect of *Clock* poly-Q genotype on timing of breeding (using standardized values for LD, OH)

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